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Lobes on *Alnus glutinosa* nodules contain a single major ribotype of *Frankia*

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This work investigated the microbial content of nodules from alders to determine how many ribotypes of *Frankia* were present and which, if any, other bacteria existed within nodes from the nodules. The bacterial content of alder nodules was investigated by 454 sequencing of 16S *rDNA* genes. Over half of the sequences were from a single ribotype of *Frankia*, with nearly all other sequences coming from the chloroplast of the host plant, and other sequences (including other ribotypes of *Frankia*) being at < 1%. It is concluded that a single ribotype of *Frankia* is the major, although not unique, bacterium present in an individual lobe from an alder nodule.

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Introduction

Many species of alders (e.g. *Alnus glutinosa*, *A. rubra*) have been planted in forestry plots to facilitate growth of the trees planted with them (Wheeler and Miller 1990) due to fixation of atmospheric nitrogen taking place in their root systems. In addition to this principal role of facilitating growth of other trees, alders have been used in land reclamation (e.g. as a biological sink for removal of heavy metals in soil which remain following open cast mining and which is being returned to agricultural/forestry use) (Roy et al. 2007).

The fixation of atmospheric nitrogen in the root system is due to the existence of *Frankia*, a nitrogen-fixing actinomycete which exists in nodules on the roots of alder trees and other actinorhizal plants; a diverse group of shrubs and woody plants. Strains of *Frankia* often show a high level of promiscuity and are often able to infect a wide range of plants species.

A mature alder tree will commonly have numerous nodules associated with its root system, and it is well-established that an actinorhizal plant can have more than one strain of Frankia found in the various nodules around its root system (Dobritsa and Stupar 1989). Although there can be infection by different strains on a single root system, the level of diversity within a particular environment may be relatively low (Kennedy et al. 2010) and may vary in a temporal manner (Anderson et al. 2013). Despite both *Alnus* and *Casuarina* plants having a similar mode of infection; via the root hair (Berry et al. 1986; Callaham et al. 1979), there were originally suggestions that *Casuarina* plants were likely to contain more than one strain of *Frankia* (Reddell and Bowen 1985), whilst *A. glutinosa* nodules contained only a single Frankia strain (Faure-Raynaud et al. 1991). More recently it has become evident that *Myrica rubra*, another actinorhizal plant where infection is via the root hair (Callaham et al. 1979) also has nodules which can contain more than one strain of *Frankia* and that the different strains can actually be relatively divergent (He et al. 2004) and that more than a single strain of *Frankia* was observed in *A. nepalensis* nodules (Dai et al. 2004). These observations regarding *Alnus nodules* were based on either PCR-RFLP (Dai et al. 2004), which relies on differences between sequences either gaining or losing a restriction enzyme digestion site, or isozyme patterns (Faure-Raynaud et al. 1991). Due to the methods involved, and their detection thresholds, it is possible that they may actually under-estimate the extent of multiple strains being present in nodules.

To date, all studies where DNA sequencing has investigated the microbial diversity of single nodules have used the Sanger method. Although it is possible to undertake large-scale sequencing investigations via this technology, the practicalities of cloning, plasmid extraction and sequencing of numerous clones means that the process can become both time-consuming and costly. The development of next generation sequencing eases this task somewhat by generating thousands of sequences within a single run, meaning that evaluation of the composition of complex ecosystems can be undertaken more readily. The work presented here is the first application of next generation sequencing to investigate the composition of the bacterial community within nodules on *Alnus glutinosa* roots in an effort to determine if there is the potential for more than one strain to inhabit a single nodule, albeit with one at a very low abundance. In addition,

rather than making an effort to target specific bacteria by concentrating on genes involved in nitrogen-fixation (e.g. Dai et al. 2004; Welsh et al. 2009) the examination extends to a broader range of bacteria by using 16S rDNA primers.

Materials and methods

Collection of nodules

Nodules were harvested from *Alnus glutinosa* plants grown in a Eutric Cambisol soil at the Henfaes Experimental Station, Abergwyngregyn, Gwynedd, Wales (53°14'N, 4°01'W) for 3 years prior to harvesting of nodules. The nodules were harvested during August 2008 and collected from soil which was approximately 5 cm below the surface. Nodules harvested had 2-3 lobes present and had an external healthy appearance. These criteria were adopted in an effort to ensure that nodules were well-developed, but to reduce the potential for nodule senescence having taken place. Following collection, nodules were stored at - 80°C until ready for DNA analysis.

Performing PCR on nodule material

Thawed nodules were surface-washed to remove bacteria loosely associated with the surface of the nodule in an attempt to reduce contamination from bacteria other than those integral to the nodule. The periderm was peeled to remove the outer layer from two nodules from different trees. Individual lobes from each of these peeled nodules were used for DNA analysis.

Lobes were ground in a microfuge tube with a mini-pestle in sterile molecular-grade water, followed by incubation at 95°C for 15 min and centrifugation at 13000 g for 10 min. The supernatant was removed and 1µl was added directly to a FastStart high fidelity PCR system master mix. Performing PCR directly on cellular material (Hofmann and Brian 1991) has been applied previously to Frankia DNA analysis (McEwan and Wheeler 1995) with the 95°C incubation rupturing even relatively robust actinomycetes such as *Frankia*. PCR was performed using the following conditions recommended by the manufacturer (Roche) for FastStart PCR for 16S rDNA amplicons for 454 sequencing: 95°C, 2 min (Hotstart) followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min with a final extension of 72°C for 7 min. The primer CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAG TTTGATCMTGGCTCAG, *E. coli* position 27, was used as the forward primer for both PCR reactions. Two different reverse primers were used – one for each of the reactions (i.e. one per nodule) being investigated, meaning that by checking the primer allowed identification of the source nodule; CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGCTG CTGCCTYCCGTA or CCATCTCATCCCTGCGTGTCTCCGACTCA GTGATACGTCTCTGCTGCCTYCCGTA, with both corresponding to *E. coli* position 357 (Caporaso et al. 2011). Primers were used at a final concentration of 400 nM and the reaction volume was 25 µl. The PCR amplicons were checked by electrophoresis on a 1% TAE agarose gel to verify successful amplification and that amplicons were around 300 bp, the approximate size predicted when using these primers.

Following size verification, the sample concentration was normalized by mixing the two PCR products in equimolar concentrations. Both replicates within the tube went through a final concentration measurement and the sample was stored at – 20°C until ready for 454 sequencing.

Quantification of the sample was carried out using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) and a CFX 96™ real-time system (Bio-Rad) to measure relative fluorescence. Concentrations were calculated using a standard curve and the reactions normalized and pooled. Agencourt® AMPure® XP beads (Beckman Coulter) were used for PCR purification following the standard manufacturer's protocol to remove unincorporated dNTPs and primer dimers. The pooled and purified libraries were re-quantified and diluted to a final concentration of 10^7 molecules/µl.

Sequencing and bioinformatical analysis

DNA sequencing was performed using a Genome Sequencer FLX, 454 Life Sciences (Roche) and following sequencing, data analysis was performed on a Dell PowerEdge T710 with Biolinux Ubuntu 10.04 base running QIIME (Caporaso et al. 2010). Raw input sequences were de-replicated and filtered according to multiplex (primer) identifiers, minimum quality score, length, ambiguous bases, homopolymer runs and primer mismatches. Operational taxonomic units (OTUs) were picked using UCLUST (Edgar 2010) at 97% identity and Ribosomal Database Project (RDP) (Cole et al. 2005) was used to assign taxonomy. For both samples, Good's coverage and rarefaction curve were determined using rich (a package run in R) for both samples to assess if the number of sequences appeared to be an appropriate representation of the population (Rossi 2011).

Results

The abundance of each sequence obtained is shown in Table 1. In both nodules, the major organism was *Frankia*, representing 58.3 and 54.0% of the total respectively. In the case of the first nodule, this *Frankia* representation was due to 2 different ribotypes being present; one constituting 57.5% of all strains and the other constituting 0.8%, with identity levels of 98%. In the second nodule, both of these ribotypes were again present (53.6% and 0.3%, respectively) but a third ribotype was also present at lower abundance (0.1%), with 99% identity to the major ribotype and 98% identity to the other.

The next most abundant sequence detected was that of the 16S *rDNA* sequence from the host plant's chloroplasts (40.6% and 45.2%, respectively). Although there was a difference in the number of sequences obtained 8267 and 1002, rarefaction curves were beginning to plateau and Good's coverage for both data sets showed that samples were representative of their respective populations, with values of 99.8% and 99.4%. All other sequences detected were present at low levels and included sequences from the following genera: detected in both nodules (*Delftia*, *Propionibacterium*, *Brevundimonas*, *Ochrobactrum*, *Burkholderia* and uncultivated members of the Lachnospiraceae); specific to nodule 1 (*Bradyrhizobium*, *Agromyces* and *Pedobacter*); or specific to nodule 2 (*Escherichia*, *Leifsonia*, *Stenotrophomonas*, *Pseudomonas*, *Rhodococcus*, *Williamsia*, *Aeromicrobium*, *Streptococcus*, *Devosia* and *Mesorhizobium*). However, in each of these cases the sequences constituted only a minor component of the microbial community, never exceeding 0.3% of the total. All DNA sequences have been submitted to the EBI database.

Discussion

This work demonstrates that the most abundant organism present in the nodules of the alder plants is indeed *Frankia*.

Table 1: Source of 16S *rDNA* sequences detected in the two *Alnus glutinosa* nodules investigated. ND = not detected

Organism detected	Sequences in nodule 1	Sequences in nodule 2
Frankia strain 1	576	4435
Frankia strain 2	8	22
Frankia strain 3	ND	9
Alder chloroplast	407	3741
Delftia	3	4
Propionibacterium	1	10
Brevundimonas strain 1	1	7
Brevundimonas strain 2	ND	1
Brevundimonas strain 3	ND	1
Ochrobactrum	1	4
Burkholderia	1	1
Bradyrhizobium	1	ND
Agromyces	1	ND
Pedobacter	1	ND
Escherichia	ND	9
Leifsonia strain 1	ND	2
Leifsonia strain 2	ND	1
Leifsonia strain 3	ND	2
Stenotrophomonas	ND	3
Pseudomonas	ND	2
Rhodococcus	ND	1
Williamsia	ND	1
Aeromicrobium	ND	1
Streptococcus	ND	1
Devosia	ND	1
Mesorhizobium	ND	1
Uncultivated Lachnospiraceae sequence 1	1	1
Uncultivated Lachnospiraceae sequence 2	ND	5
Uncultivated bacterium	ND	1
Total number of sequences	**1002**	**8267**

It also corroborates the findings of Normand et al. (1996), who reported half (5 out of 10) of the 16S rDNA sequences obtained from nodular material may be from the plant's chloroplasts.

The current findings re-iterate observations that more than a single strain of *Frankia* can occupy a single nodule, together with a low level of additional ribotypes being present. It is interesting to note that in one of the examples used here, there are three F*rankia* ribotypes detected, which is in keeping with the observation of Pokharel (2009). However, despite the presence of these additional strains, the levels at which they are present would be supportive of only one ribotype playing a major role in the physiological activities of the nodule. The observations made regarding single isozyme patterns (Faure-Raynaud et al. 1991) would also be likely in the current work, as the minor strains of *Frankia* are likely to be below detection-level threshold. However the true generic level of additional sequences from minority strains of *Frankia* requires extension beyond the current work which is based on two samples.

The final point of interest is that in general there are very few additional (non-*Frankia*) sequences detected within the nodules, with only sequences from *Delftia* ever exceeding 0.1% of the total community. It is possible that one or more of these sequences resulted from surface contamination from the nodules which was harvested during the pealing process, despite the steps taken at the start of the extraction procedure. Equally, the concept that the sequences detected were genuinely from within the nodule cannot be precluded. In either event, it is clear that levels of non-*Frankia* sources of bacteria are extremely low in the nodule – at the level which would necessitate that next generation sequencing be used to detect any such organism at a molecular level.

This suggests that the process of nodule development is not associated with any long-term co-colonisation by significant levels of other bacterial species. This observation is probably as expected, since the mode of nodule development in *Alnus* involves root hair infection (Berry et al. 1986), as opposed to the epidermal infection process described in some other actinorhizal symbioses (e.g. Miller and Baker 1985; Lui and Berry 1991). It is also interesting to note the presence of more than a single ribotype of *Frankia* in both nodules investigated here, as well as in previous work. The mechanism by which this multiple strain infection arises merits further investigation, as sampled data do not permit (i) differentiation between co-colonisation by two strains where one out-competes the other, (ii) co-colonisation but with unequal numbers at the outset or (iii) secondary colonisation of a nodule following establishment by the original ribotype.

In conclusion, the application of next generation sequencing has investigated the number of ribotypes of bacteria in two small nodules. A single *Frankia* ribotype is predominant, with at least one minor *Frankia* ribotype being found in both examples investigated. The previous issue of chloroplast contamination constituting a major number of sequences (Normand et al. 1996) was also observed here, illustrating that earlier reports were not due to errors built in to smaller sampling sizes. Moreover, the internal structure of the nodule is relatively free of any microbes other than

Frankia, with levels of other bacterial species detected being low enough to be uncertain if they are genuine occupants of the nodule or potential contaminants detected during the isolation process.

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References

Anderson MD, Taylor DL, Ruess RW. (2013) Phylogeny and assemblage composition of Frankia in *Alnus tenuifolia* nodules across a primary succcessional sere in interior Alaska. *Mol Ecol.* 22:3864-3877.

Berry AM, McIntyre L, McCully ME. (1986) Fine structure of root hair infection leading to nodulation in *Frankia-Alnus* symbioses. *Can J Bot.* 64:292-305.

Callaham D, Newcomb W, Torrey JG, Peterson RL. (1979) Root hair infection in actinomycete-induced root nodule initiation in *Casuarina*, *Myrica* and *Comptonia*. *Botanical Gazette (Chicago)*. 140:S1-S9.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Meth*. 7:335-336.

Dai YM, He XY, Zhang CG, Zhang ZZ. (2004) Characterization of genetic diversity of *Frankia* strains in nodules of *Alnus nepalensis* (D. Don) from the Hengduan Mountains on the basis of PCR-RFLP analysis of the nifD-nifK IGS. *Plant Soil*. 267:207-212.

Dobritsa SV, Stupar OS. (1989) Genetic-heterogeneity among *Frankia* isolates from root-nodules of individual actinorhizal plants. *FEMS Microbiol Lett.* 58:287-292.

Edgar RC. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* 26:2460-2461.

Faure-Raynaud M, Daniere C, Moiroud A, Capellano A. (1991) Diversity of *Frankia* strains isolated from single nodules of *Alnus glutinosa*. *Plant Soil*. 132:207-211.

Fierer N, Knight R. (2011) Global patterns of 16*S* rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA*. 108:4516–4522.

He XH, Chen LG, Hu XQ, Asghar S. (2004) Natural diversity of nodular microsymbionts of *Myrica rubra*. *Plant Soil*. 262:229-239.

Hofmann MA, Brian DA. (1991) Sequencing PCR DNA amplified directly from a bacterial colony. *BioTech*. 11:30-31.

Kennedy PG, Schouboe JL, Rogers RH, Weber MG, Nadkami NM. (2010) *Frankia* and *Alnus rubra* canopy roots: An assessment of genetic diversity, propagule availability, and effects on soil nitrogen. *Microb Ecol*. 59:214-220.

Lui Q, Berry AM. (1991) The infection process and nodule initiation in the *Frankia-Ceanothus* root nodule symbiosis: A structural and histochemical study. *Protoplasma*. 163:82-92.

McEwan NR, Wheeler CT. (1995) PCR on the Gram-positive organism *Frankia* without prior DNA extraction. *Trends Genet*. 11:168.

Miller IM, Baker DD. (1986) Nodulation of actinorhizal plants by *Frankia* strains capable of both root hair infection and intercellular penetration. *Protoplasma*. 131:82-91.

Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C, Dawson J, Evtushenko L, Misra AK. (1996) Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family Frankiaceae. *Int J Syst Bact*. 46:1-9.

Pokharel A. (2009) Diversity of *Frankia* populations in root nodules of sympatrically grown *Alnus* species. M.S. thesis, Texas State University.

Reddell P, Bowen GD. (1985) Do single nodules of Casuarinaceae contain more than one *Frankia* strain. *Plant Soil*. 88:275-279.

Rossi J-P. (2011) Rich: an R package to analyse species richness. *Diversity*. 3:112-120.

Roy S, Khasa DP, Greer CW. (2007) Combining alders, frankiae, and mycorrhizae for the revegetation and remediation of contaminated ecosystems. *Can J Bot*. 85:237-251.

Welsh AK, Dawson JO, Gottfried GJ, Hahn D. (2009) Diversity of *Frankia* populations in root nodules of geographically isolated Arizona alder trees in Central Arizona (United States). *Appl Environ Microbiol*. 75:6913-6918.

Wheeler CT, Miller IM. (1990) Current and potential uses of actinorhizal plants in Europe. In: Schwintzer CR, Tjepkema JD. (eds.) The Biology of *Frankia* and actinorhizal plants. Academic Press, Toronto, pp. 365-389.